Departement für Kleintiere, Abteilung für Radio-Onkologie

der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. med. vet. Patrick R. Kircher

Arbeit unter wissenschaftlicher Leitung von

PD Dr. med. vet. Carla Rohrer Bley, Leitung Abteilung Radio-Onkologie

SAFETY, TOLERABILITY AND PHARMAKOCINETICS PROPERTIES OF THE NOVEL TRIAZENE TriN 2755 IN TUMOR BEARING DOGS

Inaugural-Dissertation

zur Erlangung der Doktorwürde der

Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Ilektra Athanasiadi

Tierärztin

von Thessaloniki, Griechenland

genehmigt auf Antrag von

PD Dr. med. vet. Carla Rohrer Bley, Hauptreferentin

Prof. Dr. med. vet. Hanspeter Nägeli, Korreferent

2014

Aknowledgements

I dedicate this dissertation to my husband, Grigorios, whose unwavering support and motivation was incredible, even when things seemed to be impossible and difficult to handle. Without his constantly encouragement I could not have reached my goals.

“αδύνατον τον μηδέν πράττοντα πράττειν εύ”

“He who does nothing cannot do well”

Aristoteles (384-322 v. Chr.)

Inhalt

Summary in English

Vetsuisse Faculty University of Zurich (2014)

Ilektra Athanasiadi

Division of Radiation Therapy and Oncology, ehuber@vetclinics.uzh.ch

SAFETY, TOLERABILITY AND PHARMAKOCINETICS PROPERTIES OF THE NOVEL TRIAZENE TriN 2755 IN TUMOR BEARING DOGS

TriN 2755 is an alkylating antineoplastic agent for intravenous (IV) use which carries the triazene group as cytotoxic principle. The purpose of the study was to determine the maximum tolerated dose (MTD), the dose limiting toxicity (DLT), and pharmacokinetic (PK) profile of TriN 2755 in tumor bearing dogs. Additionally, tumor response was monitored and assessed after chemotherapy as a secondary goal. Treatment schedule consisted of IV administration of the drug over 20 minutes on two consecutive weeks per month for a total of three cycles. Thirty tumor bearing dogs were included in the study. The starting dose was 25 mg/kg and the escalation steps occurred in increments of 20% of the previous dose. The MTD was 74.6 mg/kg. The DLT was characterized by gastrointestinal adverse events only. The pharmacokinetic of TriN 2755 and its main metabolites in plasma and sputum are described in a two compartment model. The response rate for 19/30 dogs was 47.3% (6 partial remission, 3 stable disease) and the median progression-free interval (PFI) for the responders was 47d (range 21-450d). In summary, this study demonstrated a safe and tolerable dose of TriN 2755 for IV use in tumor bearing dogs. The adverse events were mild in severity and limited only in fatigue and gastrointestinal upset.

chemotherapy, triazene, histiocytic sarcoma, dog

Summary in German

Vetsuisse-Fakultät Universität Zürich (2014)

Ilektra Athanasiadi

Abteilung für Radio-Onkologie, ehuber@vetclinics.uzh.ch

SAFETY, TOLERABILITY AND PHARMAKOCINETICS PROPERTIES OF THE NOVEL TRIAZENE TriN 2755 IN TUMOR BEARING DOGS

TriN 2755 ist eine alkylierende, antineoplastische Substanz für intravenöse (IV) Verabreichung, das die Triazenegrouppe als zytotoxisches Prinzip trägt. Ziel dieser Studie war die Bestimmung der maximalen tolerierbaren Dosis (MTD), der Dosis-limitierenden Toxizität (DLT) und des pharmakokinetischen Profils von TriN 2755 in Hunden mit Tumoren. Die Ansprache auf die Chemotherapie wurde zusätzlich als sekundäres Ziel kontrolliert und bewertet. Der Behandlungsplan bestand aus der IV Verabreichung von TriN 2755 über 20 Minuten an zwei aufeinanderfolgenden Wochen pro Monat für drei Zyklen. Dreissig Hunden mit Tumoren wurden in die Studie eingeschlossen. Die Anfangsdosis war 25 mg/kg und die Eskalationsstufen erfolgten in 20%-Schritten der vorherigen Dosis. Die MTD war 74,6 mg/kg. Als dosis-limitierende Toxizität wurden nur gastrointestinale Nebenwirkungen beobachtet. Die Pharmakokinetik von TriN 2755 und seinen Hauptmetaboliten im Plasma und Sputum sind in einem zwei Kompartiment-Modell beschrieben. Die Ansprache-Rate für 19/30 Hunde erreichte 47,3% (6 partielle Remission und 3 stabile Erkrankung) und die mediane progressionsfreie Intervall für die Hunde, die angesprochen haben, war 47 Tage (21-450 Tage). Zusammenfassend konnte in dieser Studie gezeigt werden, dass es nebenwirkungsarme, tolerierbare Dosis von TriN 2755 für IV Verabreichung bei Hunden mit Tumoren gibt. Die Nebenwirkungen waren mild und selbstlimitierend, aus Müdigkeit und gastrointestinale Störungen bestehend.

Chemotherapie, Triazene, histiozytäres Sarkom, Hund

Manuscript

Title: Safety, tolerability and pharmacokinetic properties of the novel triazene TriN 2755 in tumor bearing dogs.*

I.Athanasiadi[1], C. Geigy[1], R.A. Hilger[2], V. Meier[1], C. Rohrer Bley[1]

1 Division of Radiation Oncology, Vetsuisse Faculty, University of Zurich, Switzerland

2 University of Essen, Clinic for Internal Medicine, Cancer Research, West German Cancer Center, Germany

Correspondence address: C. Rohrer Bley, Division of Radiation Oncology, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 258C, 8057, Zurich, Switzerland, email: crohrer@vetclinics.uzh.ch

*This study was presented in part as abstracts (oral presentation) at the Annual Conferences of the Veterinary Cancer Society in Las Vegas, NV, USA, October 2012 and Minneapolis, MN, USA, October 2013

Abbreviated title: TriN 2755 for canine malignant tumors

Abstract: TriN 2755 is an alkylating antineoplastic agent for intravenous (IV) use which carries the triazene group as cytotoxic principle. The purpose of the study was to determine the maximum tolerated dose (MTD), the dose limiting toxicity (DLT), and pharmacokinetic (PK) profile of TriN 2755 in tumor bearing dogs. Additionally, tumor response was monitored and assessed after chemotherapy as a secondary goal. The starting dose was 25 mg/kg and the MTD was 74.6 mg/kg. The DLT was characterized by gastrointestinal adverse events only. The pharmacokinetic of TriN 2755 and its main metabolites in plasma and sputum are described in a two compartment model. The response rate for 19/30 dogs was 47.3% (6 partial remission, 3 stable disease) and the median progression-free interval (PFI) for the responders was 47d (range 21-450d).

Keywords: chemotherapy, triazene, histiocytic sarcoma, dog

Introduction:

Triazenes are established alkylating chemotherapeutic compounds in clinical use in humans and dogs, which can induce apoptosis in susceptible tumors and act as antimetastatic agents.[1, 2] The triazenes drugs that are of main clinical interest include dacarbazine (DTIC) and temozolomide (TMZ). These two compounds have similar chemical, physical, antitumor and mutagenic properties.[1] TriN 2755 is an alkylating antineoplastic agent for intravenous (IV) use with a unique chemical structure, which carries the triazene group as a cytotoxic principle. Although TriN 2755 belongs to the group of triazene compounds, it differs due to its physicochemical properties as well as its potent antineoplastic activity. TriN 2755 is highly photostable and hydrophylic, showing potent dose dependent antitumor activity in a variety of human cancer xenografts in murine models including also DTIC resistant tumors.[3] Pharmacokinetic studies in rats and healthy beagle dogs demonstrated a dose dependent increase of the plasma concentration and area under the curve (AUC) of TriN 2755, following intravenously administration of the drug. TriN 2755 is well tolerated in these species

(Investigator´s Brochure, Trin Therapeutics GmbH, Düsseldorf, Germany).
A phase I clinical trial with the use of TriN 2755 was initiated in tumor bearing dogs, where no treatment existed or showing resistance to standard chemotherapy treatment. The purpose of the study was to determine the maximally tolerated dose (MTD) of TriN 2755, to look at the adverse events (AE) profile and, to correlate toxicity to pharmacokinetic profile. Additionally, tumor response was monitored and assessed as a secondary goal.

Material and Methods:

Patient Eligibility Criteria: Dogs presented to the Vetsuisse Faculty, University of Zurich, Switzerland with histologically or cytologically confirmed neoplastic disease were prospectively enrolled in this study if they were refractory to standard of care treatment, if the owner declined other treatment, or if no established standard of care existed. In order to meet inclusion criteria, dogs required adequate hematological, renal, and hepatic function and absence of any serious systemic disorder incompatible with the study. However some patients were included in the study regardless moderate hematological and chemistry changes and/or moderate clinical condition, if the owner desired a `last chance´. Prior chemotherapy treatment had to be terminated at least three weeks before entering the study. Written owner´s consent was obtained and the study was approved by the Animal Ethics Council of the Canton of Zurich, Switzerland.

Study Design and Drug Administration: This trial was a phase I dose-escalating, open-label assessment of safety and toxicity of TriN 2755 in client-owned dogs with spontaneously occurring tumors. The assessment and treatment was initially carried out on three dogs, in dose-escalating cohorts using a traditional 3+3 design.[4, 5] Escalation was performed according to Vail[5] where the dose was escalated if none of three dogs of one dose cohort developed dose-limiting toxicity (DLT). If one of the three dogs of a cohort experienced DLT, at least three more dogs were included at the same dose level. Dose escalation was stopped, if two or more dogs of a cohort of three, or an extended cohort of six patients, respectively, developed DLT; the dose of this cohort was the maximally administered dose. The maximally tolerated dose (MTD) was defined as the dose of the highest dose level in which no more than one of six dogs develops DLT.[5] The escalation steps occurred in

increments of 20% of the previous dose. Starting dose was 25 mg/kg, based on 50% of the MTD according to experimental beagle data (Investigator´s Brochure, Trin Therapeutics GmbH, Düsseldorf, Germany).[5]

The treatment schedule consisted of IV administration of the drug over 20 minutes on two consecutive weeks per month for a total of three cycles. In turn, dogs were evaluated every week for adverse events and tumor response. If no progression was noted at the end of the three cycles, treatment with TriN 2755 was continued at the same dose level but on an every two weeks schedule. TriN 2755 (Trin Therapeutics GmbH, Düsseldorf, Germany) for *in vivo* administration was provided as a powder; the powder was diluted with sterile water for injection, to obtain a concentration of 2mg/ml. The calculated dose was given as an IV infusion in parallel with 250 ml 0.9% saline over a 20-minute interval through the PhaSeal system. Supportive care (e.g. antiemetics and or antidiarrheal drugs) was administered after occurrence of AE and was provided prophylactically after the next administration of drug. Maropitan (2 mg/kg, SID, orally or subcutaneously) or Ondasentron (0.1-0.2 mg/kg, BID, orally or IV) was given against nausea and emesis, and Metronidazol (15mg/kg, BID, orally or IV) against diarrhea. Medication allowed for disease unrelated comorbidity and/or further supportive care included steroids and non-steroidal anti-inflammatory drugs (NSAIDs). Antibiotics were prescribed in case of additional infection related or unrelated to the tumor (e.g. urinary tract infection, infection of tumor). Analgesics such as tramadol or NSAID for tumor-associated pain were allowed.

Toxicity Assessment: Dogs were evaluated for toxicity weekly before each treatment, and one week after the last chemotherapy; evaluation of toxicity to determine the MTD was done after the first dose only. The AE were monitored by performing a physical examination, complete blood count (CBC) and serum biochemistry at each scheduled appointment. Owners were asked to keep a diary on animal's performance at home. Blood results and signs of AE were graded according to the VCOG-CTCAE v1.1.[6] Toxicity criteria considered as unacceptable which lead to discontinuation of the trial were defined as ≥ grade 3 toxicity, except hematological toxicities, where ≥ grade 4 considered unacceptable. The trial also included heavily pretreated patients with an advanced stage of disease,

thus a baseline status of symptoms using the same criteria was recorded for each dog before the trial started.

Tumor Response Assessment: Due to the dose-escalating nature of this trial, response to treatment was only a secondary goal. Tumor size (locoregional and systemic) was assessed at study entry, before each drug administration if the tumor was measurable without the confirmation of diagnostic imaging (e.g. peripheral lymphnodes, skin tumors), and three weeks after the last treatment by means of caliper measurements and additional diagnostic imaging according to the tumor. For intra-thoracic and intra-abdominal tumors re-evaluation (thoracic radiographs and eventually abdominal ultrasound) was also performed in the 5^{th} or 6^{th} week after the first administration of TriN 2755. Response was assessed as follows: in dogs with disseminated histiocytic sarcoma (DHS) and other solid tumors, response was measured according to the revised RECIST guideline v1.1. [7] The sum of diameters of all target lesions was calculated and reported as the baseline sum diameter. In dogs with multicentric lymphoma, criteria according to the VCOG consensus document for peripheral nodal lymphoma were used.[8] Lymph nodes were measured using calipers and the mean sum of the longest diameter of a maximum of five target lesions was calculated. According to both guidelines, responses were considered to be complete if all target lesions had disappeared (or lymph nodes were of normal size, respectively), partial (PR) if a 30% decrease of the sum of target lesion diameters occurred, or progressive (PD) if new lesions occurred or if there was an increase of at least 20% in the sum of target lesion diameters, taking as reference the smallest sum of the study. Dogs that showed neither sufficient shrinkage, to qualify for PR, nor sufficient increase to qualify for PD were considered to have stable disease (SD).[7, 8]

Pharmacokinetic Evaluation: Blood samples were evaluated at least in one dog of every dose-group before, at the end and 0.5, 1, 2, 3, and 4 hours after IV TriN 2755 infusion. Blood was collected in tubes containing EDTA. Samples were put immediately on ice and centrifuged for 10 minutes at 4°C by 2000 x g. Supernatant plasma was removed and stored at -80°C until further analysis. In addition, samples from emesis (four in total) were collected and stored at -80°C until further analysis. Plasma and emesis samples were analyzed by a validated LC-MS-HPLC method; in brief, a rapid and sensitive liquid chromatography-mass spectrometry (LC–MS) method combined with ultraviolet (UV) detection was developed and validated for the quantitative determination of TriN2755 and its

metabolites from aliquots of ethylenediaminetetra-acetic acid (EDTA) plasma. The sample extraction and cleaning-up involved a simple solid phase extraction. Chromatographic separations were achieved on a reversed phase C18 column eluted at a flow-rate of 1.00 ml/min on a gradient of formic acid, methanol and acetonitrile. The overall cycle time of the method was 40 minutes. The monitored masses using electrospray ionisation were 193, 198, 214, 215 and 256 (m/z) for TriN2755 and its putative metabolites, respectively. For UV detection, a wavelength of 300 nm was used. The calibration curves were linear over the range of 3.00–400 ng/ml with the lower limit of quantification (LLQ) validated at 3.00 ng/ml for MS detection and between 400 ng/ml up to 100 µg/ml using UV detection at 300 nm. The results obtained during the validation of this method fulfill all requirements and recommendations, generally accepted for bioanalytical studies.[9-11] The within-run and between-run precisions, also at the levels of LLQ and upper limit of quantification (ULQ), ranged between 3.0 and 10.8% (as [%] coefficient of variation (cv)), while the accuracy (as [%]bias) ranged from +1.1 to -8.6%. The within-run and between-run precisions in this range were within 4.99%, while the accuracy ranged from 95.8 to 100.3%. The method was successfully applied to samples derived from this clinical phase I study.

Statistical analysis: Plasma pharmacokinetic parameters, area under the curve (AUC), maximum concentration (Cmax), time to maximum concentration (Tmax), and elimination half-life (t1/2) for TriN 2755 and its metabolites were calculated using a two-compartment disposition model (program TOPFIT 2.0).[12] The linear-logarithmic trapezoidal method was used to calculate AUC, and apparent t1/2 was calculated by linear least squares regression after logarithmic transformation of the terminal concentrations.

Results:

Patient Characteristics: A total of 30 client-owned dogs were included in the study between April 2011 and July 2013. Patient's population consisted of dogs of various breeds; the most common breed was the Bernese Mountain Dog (BMD) (n=13). Other breeds included are shown in Table 1. Median age upon study entry was 8.3 years (range 4.3-14.6 years) and median weight 35.7 kg (range 8.7-60.1 kg). Nine dogs were intact (3 females, 6 males), eleven were castrated males and ten spayed females.

Different types of tumors were included; the most common primary tumor was histiocytic sarcoma (HS) (n=14). Thirteen dogs had the disseminated form (DHS) and one had the localized form (LHS). The most common anatomical location for the DHS was the lung (n=8), followed by spleen (n=3), tracheobronchial lymphnodes (n=2), liver (n=2), mediastinal and abdominal lymphnodes, eye, bone and, kidney. The dog with the LHS had the tumor in its right elbow region. Other tumors included are shown in Table 1. More than half of the dogs had not received any prior treatment (n=18), whereas twelve dogs of the study had either chemotherapy (6 with malignant lymphoma and 1 with disseminated HS), surgery (2 splenic hemangiosarcomas) or radiation therapy (the two malignant melanomas) before receiving TriN 2755. The dog with the localized LHS had radiation therapy and received TriN 2755 as additional chemotherapy. All six dogs with malignant lymphoma showed multicentric involvement stage III-V, substage a or b disease and clinical resistance towards several previous chemotherapy protocols.

Drug Administration: Patients were divided into eight different dose groups from 25 mg/kg to 89.5 mg/kg with increments of 20%. The groups consisted of three dogs; one of the three dogs from the fifth group experienced DLT and three additional dogs were added to this group. None of the three additive dogs experienced any DLT, thus the dose escalation was continued. At the MTD level three confirmatory patients were added.

33% (10/30) of the enrolled patients completed the three cycles as planned and two of these continued treatment due to partial remission or stable disease; 2 patients with DHS had 18 and 9 applications of TriN 2755, respectively. Nineteen dogs received TriN 2755 only 2-4 times because of tumor progression or because of owner´s wish to discontinue (e.g. dogs in end-stage disease at entry without signs of improvement). This applied to the patients that had 2 applications of TriN 2755. One dog of the study received TriN 2755 only once because it died 8 days after the first chemotherapy; the cause of death in this case was unrelated to the tumor or the chemotherapeutic agent. 8/30 dogs received concurrent NSAIDs and 11/30 received steroids (oral prednisolone). Of the six lymphoma patients only one received steroids during the treatment with TriN 2755.

Toxicity Assessment: The toxicity was assessed after the first administration of TriN 2755. Over the 12-week period, overall side effects were mild and rarely dose-limiting, as summarized in Table 2.

Hematological and biochemical parameters:

Hematological and biochemical changes were not observed in any of the patients receiving the study drug. 14/30 dogs had already hematological and biochemical abnormalities at the time of inclusion either due to concurrent disease or due to causes related to the tumor.

Gastrointestinal effects:

Main gastrointestinal AE were anorexia, diarrhea, nausea and vomiting beginning within 24 h after the end of the infusion of TriN 2755. 3/30 dogs of the study experienced dose-limiting toxicities due to diarrhea and vomiting. When occurrence of gastrointestinal AE was detected after the first drug administration, supportive care was administered in 24/30 dogs. Not all AE were considered to be related to TriN 2755 administration; in some dogs, observed toxicities were rather related to disease progression or to treatment-unrelated concurrent morbidities.

Other effects:

Grade 1-2 fatigue was observed in 19/30 dogs after the first administration of TriN 2755.

Table 2 provides a summary of the adverse events noted after the first administration of the study drug approximately within 24h.

Maximally Tolerated Dose: TriN 2755 was well tolerated up to a dose of 74.6 mg/kg. At the next higher dose level of 89.5 mg/kg, two of three dogs showed dose-limiting gastrointestinal toxicity grade 3, therefore the cohort study stopped. MTD for TriN 2755 application was determined to be 74.6 mg/kg.

Tumor Response Assessment: Preliminary antitumor activity was examined only in patients that have completed the first cycle and received at least 3 times TriN 2755. In addition, one dog (patient 29) with metastatic lung adenocarcinoma receiving 4 times TriN 2755 was excluded from the response-analysis due to difficulties in evaluating the tumor because of an almost continuous production of free fluid in the thorax; therefore, the response was evaluated in 19/30 dogs. At the end of all TriN 2755 treatments (range 3-18), 6 (33%) dogs showed PR, 3 (16%) dogs SD, and 10 (52%) PD.

From the dogs with PR one was a BMD with DHS (18 administrations of TriN 2755, PFI 450 days), four dogs had multicentric lymphoma Stage IV-V, substage a-b (3-4 administrations of TriN 2755, PFI ranged 21-51 days) and one dog had subcutaneous malignant melanoma (3 administrations of TriN 2755, PFI 74 days).

All three dogs with SD disease completed the three cycles of TriN 2755 (treatments ranged from 6 to 9); one BMD with DHS (splenic form, PFI 139 days), one dog with LHS (PFI 117 days) and one dog with gastric adenocarcinoma (PFI 126 days).

6/10 dogs with PD were BMD with DSH; all dogs completed three cycles of TriN 2755 (median PFI 78.5 days, range 63-91 days).

Table 1 provides a summary of the patients and the response by cohort.

Pharmacokinetic of Plasma: The pharmacokinetic of TriN 2755 and its main metabolites in this infusion protocol is well described with a two compartment disposition model. An example is given in Figure 1; one dog of cohort 8 was treated with 89.6mg/kg TriN 2755. TriN 2755 and its main metabolites M1, M3 and M4 were analyzed within 4 hours after start of the slow bolus injection. By using a two compartment model, extrapolation to infinity was possible. AUC as well as Cmax of the given drug TriN 2755 increased in a linear manner with dose (r^2 = 0.92 and 0.86, respectively). We obtained a higher deviation from linearity in AUC calculation for the main metabolites M1 and M4, and even a worse correlation for Cmax calculation. Tmax of TriN 2755 was obtained at the end of infusion (about 20 min), the calculated Tmax for M1 was 0.82 +/- 0.08 h (mean +/- SD), about 30 min after end of infusion. The calculated Tmax of metabolite M4 was 1.64 +/- 0.49 h, more than 75 min after end of infusion. Terminal half-lives were 0.60 +/- 0.18 h for TriN 2755, 0.82 +/- 0.09 h for M1, and 1.34 +/- 0.43 h for M4, respectively. PK calculations for metabolite M3 were not possible in all cases. Therefore, single values are not presented (Table 3).

Analysis of sputum: 6 dogs of the study vomited orange/red fluid 2-6 hours after the administration of the study drug. All these dogs received TriN 2755 at a dose equal or more than 62.2 mg/kg. Four samples of sputum from 3 dogs were collected for further investigation. 2/4 samples were from the same dog (patient 21); collected in the first 2 h and the second 5.5 h after the administration of TriN 2755. The pH for the first sample was 7.6 and for the second 2.57. The

other two samples were collected from 2 different patients (patient 23, 26) and at the same time a blood sample was drawn; the pH of the sputum from the patient 23 and 26 were 7 and 7.7, respectively. TriN 2755 and its metabolites were detected in all four sputum samples.

Discussion:

According to this phase I trial, the maximum tolerated IV dose of TriN 2755 in dogs was determined to be 74.6 mg/kg. The toxicities related to the drug were in general mild in severity and include fatigue and gastrointestinal upset; vomitus, anorexia, nausea and diarrhea (Table 2). It is described that dogs receiving Dacarbazine and Temozolamide show primarily hematological, and gastrointestinal AE.[13-15] However none of the dogs in this study experienced any hematological changes related to the drug administration during or at the end of the study.

The DLT is characterized by gastrointestinal AE and it did not exceed grade III. Based on the results of this study 3 patients in total experienced DLT; two dogs (patient 15, 26) experienced grade III diarrhea and one dog (patient 27) showed grade III vomitus.

Cancer patients in a human phase I study tolerated TriN 2755 intravenously up to a total dose of 6000mg. Only grade 1-2 fatigue, vomitus and anorexia were observed in human patients receiving doses of TriN 2755 ranged between 25 and 6000 mg in total. None of the humans patients experienced hematological toxicity.[16] In contrast, dosages of TriN 2755 higher than 74.6 mg/kg (which correlates to 3000-4500mg in total) given to our canine patients resulted in grade 3 gastrointestinal AE. Hence, the tolerated dose in dogs seems to be lower than in man. Similar to Dacarbazine, TriN 2755 is an alkylating agent activated in the liver by CYP 450 enzymes.[1] M1 and M4 are the main metabolites of TriN 2755. While M1 is still carrying the triazene group, M3 and M4 are the anilines obtained after enzymatic demethylation of the triazene group of TriN 2755 and M1 respectively.[17] The inactive metabolite M4 is formed either after enzymatic demethylation of the triazene group of M1 via Cyp1A1, CYP1A2, and CYP2E1 (analogous to dacarbazine[18], data not shown) or by hydrolysis of metabolite M3 (Figure 1).

In humans and dogs, the extent of formation of the active metabolite M1 from TriN 2755 is higher than 50%. The inactive aniline metabolite M3 is formed in humans and dogs to negligible amounts

(less than 1% and 0.1%, respectively; data not shown). Whereas the aniline metabolite M4 is further metabolized via N-acetylation in humans (data not shown), the absence of N-acetyltransferases in dogs[19, 20] leads to fundamental differences in the pharmacokinetic profile of M4 between human and dog. By comparing the PK profile of TriN 2755 and its metabolites in dogs and humans, there are 10fold higher concentrations of the aniline metabolite M4 of TriN 2755 detectable in dogs after a comparable dose of 74.6 mg/kg in dogs (absolute dose = 3453 mg) and 73.2 mg/kg (flat dose = 6000 mg) in humans. This might be an explanation for the decreased tolerance of TriN 2755 in dogs (Figure 2). M4 is the suspected metabolite being responsible for AE. Further *in vitro* studies are necessary to explore this hypothesis.

Six dogs of this study receiving a dose of TriN 2755 equal or more than 62.2mg/kg vomited a red/orange fluid. Four sputum-samples from three of these six dogs were further evaluated; TriN 2755 and its main metabolites were detected in all four samples and the pH for 3/4 sputum-samples ranged between 7.0-7.7. These results might suggest that the colored sputum is hepatic bile and TriN 2755 and its metabolites enter the hepatobiliary circulation, considering that the normal hepatic bile of the dogs has a color golden yellow to orange and the pH ranges between 7.0- 7.8.[21] Further investigation is needed to verify this hypothesis.

This study was a dose-escalating phase I clinical trial; therefore, the response was only a secondary goal. Although most of the patients were treated with TriN 2755 at a dose lower than the MTD, which is one of the limitations of a phase I clinical trial using the "3+3" design [4], and suffered from tumors that are not easy to treat (e.g. disseminated histiocytic sarcoma, gastric adenocarcinoma, subcutaneous malignant melanoma), the responses that were registered in this study are promising.

Twelve dogs from our study were BMDs having DHS. DHS, previously known as malignant histiocytosis, is a malignancy of histiocytic cell origin.[22-24] DHS arises from the dendritic antigen-presenting cells.[24-26] Bernese Mountain Dogs, Flat-coated Retrievers, Labrador Retrievers and Rottweilers are breeds that are predisposed to HS, with the BMD showing a heritable predisposition to DHS.[24, 26-32] DHS is a rapidly progressing multisystemic disease with a poor prognosis.[23] Therefore, the recommended treatment is systemic chemotherapy.[22] In one study Lomustine (CCNU) was given in 59 dogs with HS. The response rate was 46% and the median survival time (MST) for the dogs that

responded was 172 days. The same study showed that dogs with splenic involvement had a worse prognosis with a MTS of 58 days.[28] The course of the disease for the BMDs is referred to be even worse than other breeds according to two studies, giving a MST of between 30 and 49 days.[29, 30] Remarkably, one BMD with DHS showed partial remission for 450 days, one BMD had disease stabilization for 139 days, and a Golden Retriever for 117 days, indicating that TriN 2755 might have antitumor activity against malignant tumors of histiocytic origin.

In summary, this study demonstrated a safe and tolerable dose of TriN 2755 for IV use. The AE were mild in severity and limited in fatigue and gastrointestinal upset. The response rates of this phase I clinical trial showed promising advantages of the use of this triazene, but a phase II clinical trial is needed to determine the efficacy of this drug.

Acknowledgements:

We thank the Albert-Heim-Stiftung for financial support of the study.

References:

1. Marchesi F, Turriziani M, Tortorelli G, Avvisati G, Torino F and De Vecchis L. Triazene compounds: mechanism of action and related DNA repair systems. *Pharmacological Research*. 2007; **56**: 275-287.
2. Giraldi T, Sava G, Cuman R, Nisi C and Lassiani L. Selectivity of the antimetastatic and cytotoxic effects of 1-p-(3,3-dimethyl-1-triazeno)benzoic acid potassium salt (+/-)-1,2-di(3,5-dioxopiperazin-1-yl)propane, and cyclophosphamide in mice bearing Lewis lung carcinoma. *Cancer Research*. 1981; **41**: 2524-2528.
3. Bausch N, Baltes S, Fiebig H, Beckers T and Barth W. TriN 2755: Antitumor activity of a new cytotoxic therapeutic of the triazene class. *Molecular Cancer Therapeutics*. 2009; **8**: A144.
4. Le Tourneau C, Lee JJ and Siu LL. Dose escalation methods in phase I cancer clinical trials. *Journal of National Cancer Institute*. 2009; **101**: 708-720.
5. Vail DM. Cancer clinical trials: development and implementation. *Veterinary Clinics of North America Small Animal Practice*. 2007; **37**: 1033-1057.
6. Veterinary cooperative oncology group - common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. *Veterinary and Comparative Oncology*. 2011.
7. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *European Journal of Cancer*. 2009; **45**: 228-247.
8. Vail DM, Michels GM, Khanna C, Selting KA and London CA. Response evaluation criteria for peripheral nodal lymphoma in dogs (v1.0)-a Veterinary Cooperative Oncology Group (VCOG) consensus document. *Veterinary and Comparative Oncology*. 2010; **8**: 28-37.

9. Vinod P. Shah KKM, Shrikant Dighe, Iain J. McGilveray, Jerome P. Skelly, Avraham Yacobi, Thomas Layloff, C. T. Viswanathan, C. Edgar Cook, R. D. McDowall, Kenneth A. Pittman, Sidney Spector. Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies. *Pharmaceutical Research*. 1992; **9**: 588-592.
10. Vinod P. Shah KKM, Shrikant Dighe, Iain J. McGilveray, Jerome P. Skelly, Avraham Yacobi, Thomas Layloff, C. T. Viswanathan, C. Edgar Cook, R. D. McDowall, Kenneth A. Pittman, Sidney Spector. Analytical methods validation: Bioavailability, bioequivalence, and pharmacokinetic studies. *Journal of Pharmaceutical Sciences* 1992; **81**: 309-312.
11. U.S. Department of Health and Human Services FaDA, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Guidance for industry: bioanalytical method validation. edn., 2001: 1-22.
12. Heinzel G WR, Thomann P. Topfit Version 2.0: Pharmacokinetic and Pharmacodynamic Data Analysis System for the Pc/Book and Disk. Stuttgart, Germany, Gustav Fischer Verlag/ VCH Publishers, 1993.
13. Dervisis NG, Dominguez PA, Sarbu L, Newman RG, Cadile CD, Swanson CN, et al. Efficacy of temozolomide or dacarbazine in combination with an anthracycline for rescue chemotherapy in dogs with lymphoma. *Journal of American Veterinary Medical Association*. 2007; **231**: 563-569.
14. Dervisis NG, Dominguez PA, Newman RG, Cadile CD and Kitchell BE. Treatment with DAV for advanced-stage hemangiosarcoma in dogs. *Journal of American Animal Hospital Association*. 2011; **47**: 170-178.
15. Griessmayr PC, Payne SE, Winter JE, Barber LG and Shofer FS. Dacarbazine as single-agent therapy for relapsed lymphoma in dogs. *Journal of Veterinary Internal Medicine*. 2009; **23**: 1227-1231.
16. R. A. Hilger, T. Wegener, H. Richly, G. Ludwig, K. Mross and Scheulen ME. An ongoing Phase I Pharmacokinetic (PK) study of the safety and tolerability of TriN 2755 in patients with advanced solid tumors: a study in cooperation with the central european society for anticancer drug research (CESAR-EWIN). In: *CESAR Annual Meeting 2011,* 1 edn., Greifswald, Germany, 2011: 60.
17. Lütscher N, T. Wegener, S. Hönes, H. Richly, G. Ludwig, M. E. Scheulen, et al. Antitumor in vitro activity of TriN 2755 and its metabolites/derivates. In: *Program and Abstract Book: CESAR Annual Meeting 2012, June 28 – 30 2012, Essen,* 1 edn., Essen, Germany, 2012: 60.
18. Reid JM, Kuffel MJ, Miller JK, Rios R and Ames MM. Metabolic activation of dacarbazine by human cytochromes P450: the role of CYP1A1, CYP1A2, and CYP2E1. *Clinical Cancer Research*. 1999; **5**: 2192-2197.
19. Gregus Z, Watkins JB, Thompson TN, Harvey MJ, Rozman K and Klaassen CD. Hepatic phase I and phase II biotransformations in quail and trout: comparison to other species commonly used in toxicity testing. *Toxicology and Applied Pharmacology*. 1983; **67**: 430-441.
20. Neis JM, Yap SH, Van Gemert PJ, Roelofs HM and Henderson PT. Mutagenicity of five arylamines after metabolic activation with isolated dog and human hepatocytes. *Cancer Letters*. 1985; **27**: 53-60.
21. Center SA. Diseases of the gallbladder and biliary tree. *The Veterinary Clinics of North America Small Animal Practice*. 2009; **39**: 543-598.
22. Coomer AR and Liptak JM. Canine histiocytic diseases. *Compendium Continuing Education Vet*. 2008; **30**: 202-204, 208-216; quiz 216-217.
23. Fulmer AK and Mauldin GE. Canine histiocytic neoplasia: an overview. *The Canadian Veterinary Journal*. 2007; **48**: 1041-1043, 1046-1050.
24. Moore PF. A review of histiocytic diseases of dogs and cats. *Veterinary Pathology*. 2014; **51**: 167-184.
25. Moore PF, Affolter VK and Vernau W. Canine hemophagocytic histiocytic sarcoma: a proliferative disorder of CD11d+ macrophages. *Veterinary Pathology*. 2006; **43**: 632-645.
26. Affolter VK and Moore PF. Localized and disseminated histiocytic sarcoma of dendritic cell origin in dogs. *Veterinary Pathology*. 2002; **39**: 74-83.
27. Craig LE, Julian ME and Ferracone JD. The diagnosis and prognosis of synovial tumors in dogs: 35 cases. *Veterinary Pathology*. 2002; **39**: 66-73.

28. Skorupski KA, Clifford CA, Paoloni MC, Lara-Garcia A, Barber L, Kent MS, et al. CCNU for the treatment of dogs with histiocytic sarcoma. *Journal of Veterinary Internal Medicine*. 2007; **21**: 121-126.
29. Abadie J, Hedan B, Cadieu E, De Brito C, Devauchelle P, Bourgain C, et al. Epidemiology, pathology, and genetics of histiocytic sarcoma in the Bernese mountain dog breed. *Journal of Heredity*. 2009; **100 Suppl 1**: 19-27.
30. Rosin A, Moore P and Dubielzig R. Malignant histiocytosis in Bernese Mountain dogs. *Journal of American Veterinary Medical Association*. 1986; **188**: 1041-1045.
31. Padgett GA, Madewell BR, Keller ET, Jodar L and Packard M. Inheritance of histiocytosis in Bernese mountain dogs. *Journal of Small Animal Practice*. 1995; **36**: 93-98.
32. Dobson JM. Breed-predispositions to cancer in pedigree dogs. *ISRN Veterinary Science*. 2013; **2013**: 23.

Table 1. Patients and response by cohort

Cohort	TriN 2755 (mg/kg)	Number of dog	Breed	Tumor	Number of treatments	Tumor Response	PFI
1	25	1	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	6	PD	70d
		2	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	6	PD	71d
		3	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	2	NE	
2	30	4	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	18	PR	450d
		5	Mix breed	Lymphoma	2	NE	
		6	Dachshund	Lymphoma	1	NE	
3	36	7	Mixed breed	Gastric Adenocarcinoma	6	SD	126d
		8	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	2	NE	
		9	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	6	PD	91d
4	43.2	10	Greater Swiss Mountain Dog	Splenic hemangiosarcoma	2	NE	
		11	St. Bernard	Renal Adenocarcinoma	2	NE	
		12	Mixed Breed	Oral melanoma	3	NE	
5	51.8	13	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	9	SD	139d
		14	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	6	PD	86d
		15	Mixed breed	Disseminated Histiocytic Sarcoma	2	NE	
		16	Mixed breed	Lymphoma Stage Vb	4	PR	43d
		17	Mixed breed	Gastric Adenocarcinoma	2	NE	
		18	Golden Retriever	Localized Histiocytic Sarcoma	6	SD	117d
6	62.2	19	Golden Retriever	Lymphoma Stage IVa	4	PR	51d
		20	Golden Retriever	Lymphoma Stage IVa	3	PR	21d
		21	Jack Russel Terrier	Lymphoma Stage IVb	3	PR	23d
7	74.6	22	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	6	PD	89d
		23	Mixed breed	Splenic hemangiosarcoma	4	PD	60d
		24	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	6	PD	63d
		28	Mixed breed	Subcutaneous malignant melanoma	3	PR	74d
		29	Mixed breed	Metastatic lung Adenocarcinoma	4	NE	
		30	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	2	NE	
8	89.5	25	Giant Schnauzer	Renal hemangiosarcoma	4	PD	55d
		26	Bernese Mountain Dog	Disseminated Histiocytic Sarcoma	4	PD	39d
		27	Bernese Mountain Dog	Lung Adenocarcinoma	4	PD	56d

PR, Partial Remission; SD, Stable Disease; PD, Progressive Disease; NE, Not Evaluated

Appendix 1: Table 1

Table 2. Dose and toxicity by cohort

Cohort	TriN 2755 (mg/kg)	Dogs treated	Number of treatments	Dose-limiting adverse events	Toxicity after the first administration (grade)				
					Hematological	Vomitus/ Nausea	Anorexia	Diarrhea	Fatigue
1	25	3	6/6/2	0			1	1	1-2
2	30	3	18/2/1	0			1	1	1-2
3	36	3	6/2/6	0		1			1
4	43.2	3	2/2/2	1 (diarrhea)					1
5	51.8	6	9/6/2/4/2/6	0		1-2	1-2	1-3	1
6	62.2	3	4/3/3	0		2	1	1	1
7	74.6	6	6/4/6/3/4/2	0		1	1	1	1
8	89.5	3	4/4/4	2 (diarrhea, vomitus)		2-3	1	2-3	1

Table 3. Pharmacokinetic of TriN 2755 and its main metabolites in plasma

	TriN 2755				M1				M4			
Dose [mg/kg]	Cmax [µg/ml]	AUC [µg/ml*h]	t1/2 [h]	Tmax [h]	Cmax [µg/ml]	AUC [µg/ml*h]	t1/2 [h]	Tmax [h]	Cmax [µg/ml]	AUC [µg/ml*h]	t1/2 [h]	Tmax [h]
25	13.7		0.41		49.1		0.6		22.8		1.16	
25	46.1	92	0.41	0.38	47.8	92	0.47	0.78	26.7	127	2.16	1.3
25	171	105	0.56	0.33	55.3	99.9	1.42	0.78	26.2	86	1.42	1.42
30	215	106	0.49	0.33	112	185	0.42	0.68	43.5	112	1.41	1.1
36	194	105	0.52	0.33	111	209	1.42	0.82	40	141	1.46	1.51
53.2	221	155	0.78	0.38	72.2	174	0.79	0.81	38.7	160	0.72	1.68
62	271	227	0.44	0.38	84.4	169	1.43	0.87	49.3	165	1.42	1.36
74.6	456	374	0.67	0.38	145	354	0.78	0.97	69.3	311	0.89	1.76
89.5	588	442	0.94	0.38	109	365	1.03	0.81	35.6	232	1.22	2.63

Appendix 1: Table 3

Appendix 2: Figure 1

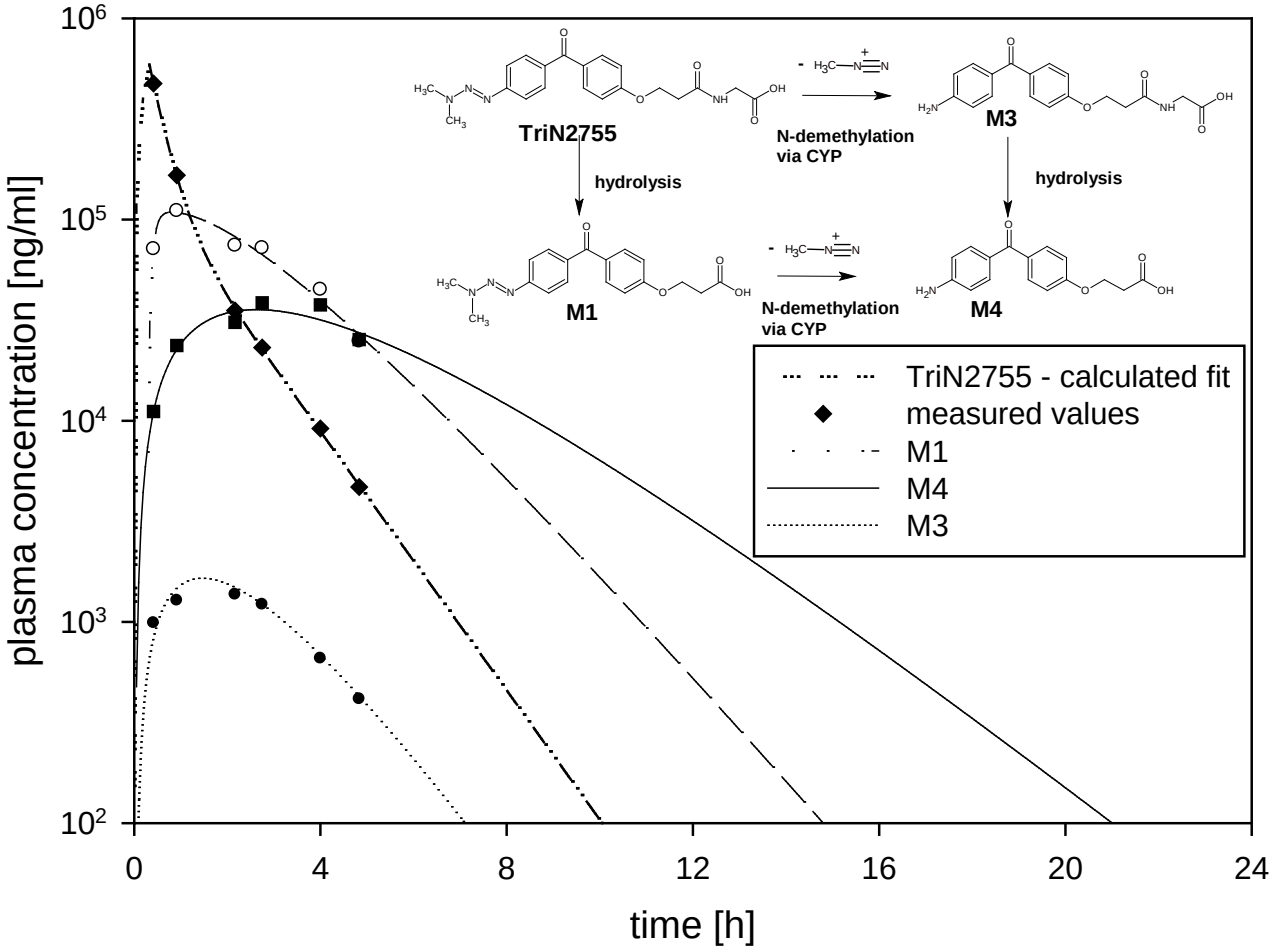

Figure 1. Representative diagram of the pharmacokinetic profile of TriN 2755 and its main metabolites M1, M3 and M4 after a dosage of 89.6 mg/kg TriN 2755. Dots represent measured values, the continuous lines the calculated fits.

Appendix 2: Figure 2

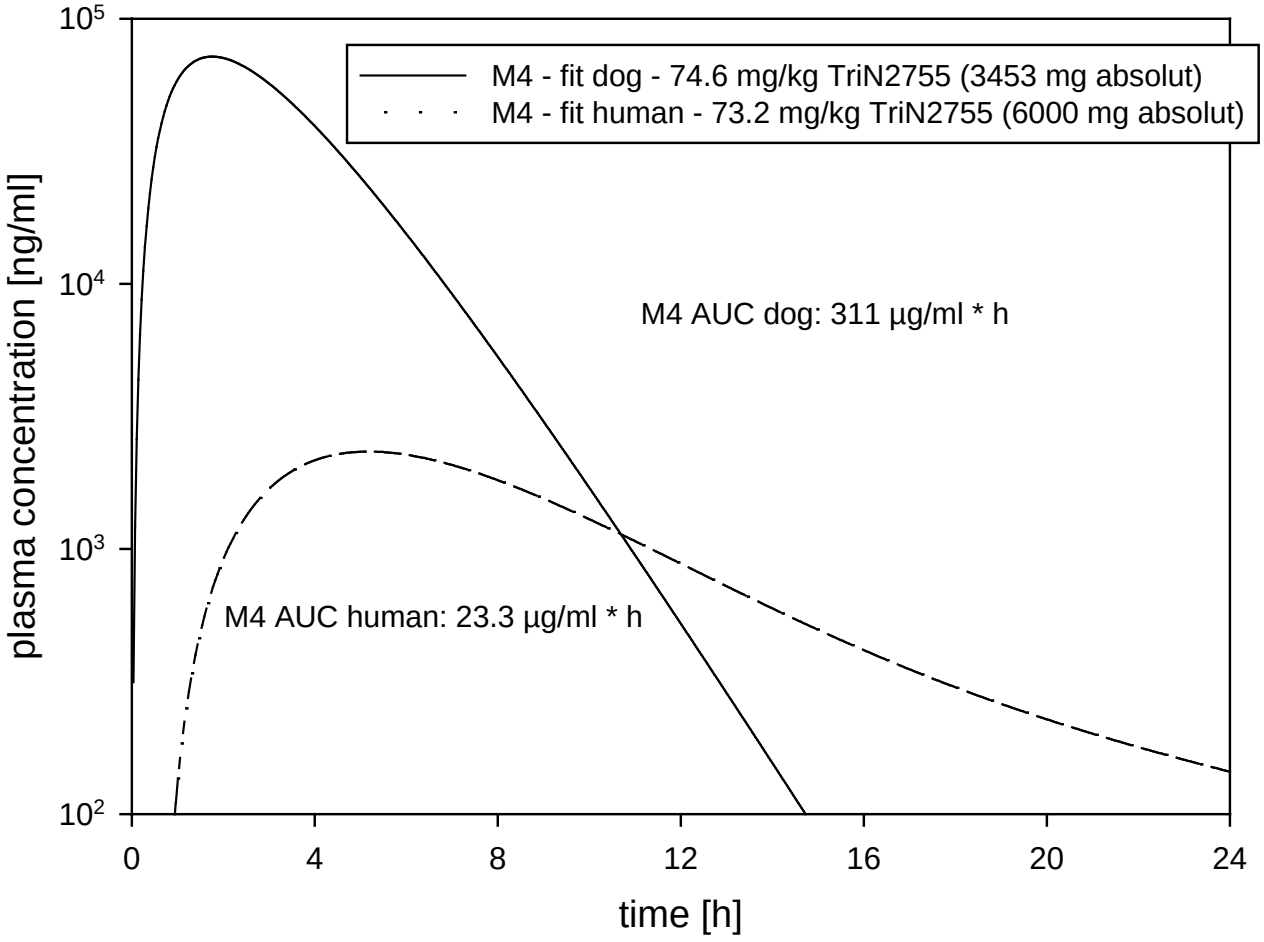

Figure 2. The fitted concentration vs. time plots and the calculated AUCs (area under the curve) of the metabolite M4 formed in human and dog.

Appendix 3: Patients-data

	Number of dog - KG	Age (years)	Gender	Weight (kg)	Anatomical location	Previous treatment	NSAIDs/ Steroids
1	1- 2179345 2- 2181283 3- 2182760	7.1 9.1 7.2	Female, castrated Female, castrated Male, castrated	36.6 35.1 42.5	Lung Lung Lung	- - Chemotherapy	- Steroids Steroids
2	4- 2183314 5- 2181908 6- 2109134	9 12 13	Female, castrated Male, castrated Male, castrated	44.1 33.4 10	Lung, eye Peripheral & abdominal lnn, spleen, liver Peripheral lymphnodes (lnn)	- Chemotherapy Chemotherapy	NSAIDs Steroids Steroids
3	7- 2186722 8- 2187306 9- 2189038	10.7 7 6.8	Female, castrated Male, castrated Male, intact	26.2 36.3 43	Stomach Lung, mediastinal lnn Lung, mediastinal lnn, spleen, left humerus	- - -	- Steroids NSAIDs
4	10- 2176564 11- 2203242 12- 2189484	9.2 14 4.3	Female, castrated Female, castrated Male, castrated	36.1 13 60	Spleen Mandibula Kidney, lung metastasis	Splenectomy RT, chemotherapy -	NSAIDs NSAIDs -
5	13- 2189775 14- 2191039 15- 2169922 16- 2188430 17- 2195402 18- 2195864	6 6 8.3 12 8.2 9	Male, castrated Male, castrated Male, castrated Male, castrated female, castrated Male, castrated	45.5 45 43 20 12.3 32.6	Spleen Left kidney, spleen, lung, abdominal lnn Lung, tracheobronchial lnn Peripheral nn, spleen, skin Stomach Right elbow region	- - - Chemotherapy - Surgery	NSAIDs/Steroids Steroids - Steroids - NSAIDs
6	19- 2193155 20- 2182384 21- 2190136	4.3 8.3 8.3	Female, intact Female, intact Male, castrated	31.1 26.3 8.8	Peripheral & abdominal lnn, spleen Peripheral lnn, spleen Peripheral lnn, spleen, liver	Chemotherapy Chemotherapy Chemotherapy	- - -
7	22- 2145326 23- 2198914 24- 2200630 28- 2096881 29- 2183192 30- 2116404	5 11 5 14.5 12.9 8.2	Male, castrated Male, intact Male, intact Female, castrated Female, castrated Male, intact	46.3 28 44.3 28.8 12.1 41.8	Lung Spleen Liver Right axillary region, lung metastasis Lung Lung, abdominal lnn, lift kidney, liver	- Surgery - RT, chemotherapy - -	- NSAIDs - Steroids NSAIDs Steroids
8	25- 2200670 26- 2200955 27- 2123925	7.8 6.7 9.5	Male, intact Male, intact Female, intact	41.8 36.8 49	Kidney Lung Lung	- - -	- - -

Curriculum Vitae

Vorname Name	Ilektra Athanasiadi
Geburtsdatum	19 April 1982
Geburtsort	Thessaloniki
Nationalität	Griechenland

09/1994-06/2000	**Schulausbildung** Grundschule-Gymnasium-Lyzeum, Sparta, Griechenland
06/2000	**Höchster Schulabschluss** Abitur von der allgemeinen gymnasialen Oberstufe, Sparta, Griechenland
09/2000-09/2006	**Studium** Veterinärmedizin, Aristoteles Universität, Thessaloniki, Griechenland
01/12/2006	Abschlussprüfung DVM, Aristoteles Universität, Thessaloniki, Griechenland
08/2011-04/2014	Anfertigung der Dissertation am Departement für Kleintiere, Direktor Prof. Dr. Patrick R. Kircher, unter Leitung von Frau PD Dr. med. vet. Carla Rohrer Bley, Leitung Abteilung für Radio-Onkologie, Vetsuisse-Fakultät Universität Zürich
12/2006-10/2008	Vollzeitstelle als Tierärztin in der Überweisungskleintierklinik von Dr. Ignatios Liapis (www.vetclinik.gr), Athen, Griechenland
01/2010-01/2011	Internship an der Kleintierklinik der Tierärztlichen Hochschule Hannover, Deutschland
08/2011-12/2013	Assistenzärztin in der Abteilung für Radio-Onkologie am Departement für Kleintiere, Vetsuisse-Fakultät Universität Zürich, Schweiz
02/2013-bisher	Notfalltierärztin am Departement für Kleintiere, Vetsuisse-Fakultät Universität Zürich, Schweiz